Thesis Synopsis

Characterization of the *cis* and *trans* acting factors that influence p53 IRES function

p53 is a nodal tumor suppressor protein that acts as a major defense against cancers. Approximately 50% of human tumours have mutations in p53 gene. Among its myriad features, the most distinctive is the ability to elicit both apoptotic death and cell cycle arrest. p53 has several isoforms. Most of them are produced by either internal promoter activity of the gene or alternate splicing of the pre-mRNA. Apart from these mechanisms, p53 mRNA has also been shown to be translated into two isoforms, the full-length p53 (FL-p53) and a truncated isoform ΔN-p53, which acts as a dominant-negative inhibitor of FL-p53.

Under conditions of cellular stress, the canonical mode of translation initiation is compromised. To maintain the synthesis of proteins important for cell survival and cell-fate decisions, a subset of cellular mRNAs utilizes a non-canonical mode of translation initiation. The 5' untranslated region of these mRNAs are highly structured and function as Internal Ribosome Entry Site (IRES). Previously, from our laboratory it has been shown that translation of p53 and its N-terminally truncated isoform ΔN-p53 can be initiated by IRES mediated mechanism. IRES mediated translation of ΔNp53 was maximum at G1-S phase but that of FL-p53 was maximum at the G2-M phase. Interestingly in case of a human genetic disorder X-linked dyskeratosis congenita (X-DC), aberrant IRES mediated p53 translation has been reported. It has also been reported that during oncogenic induced senescence (OIS) a switch between cap-dependent to IRES meditated translation occurs in p53 mRNA. From our laboratory, we have also demonstrated that polypyrimidine tract binding protein (PTB) positively regulates the IRES activities of both the p53 isoforms by shuttling from nucleus to

the cytoplasm during genotoxic stress conditions. It is very important to understand how these two isoforms are regulated and in turn control the cellular functions.

In the first part of the thesis, to investigate the importance of the structural integrity of the *cis* acting elements within p53 RNA, we have compared the secondary structure of the wild-type RNA with cancer-derived silent mutant p53 RNAs having mutations in the IRES elements such as L22L (CTA to CTG) a natural cancer mutation and Triple Silent Mutation (mutations were present at the wobble position of codon 17, 18, 19). These mutations result in the conformational alterations of p53 IRES RNA that abrogates the IRES function *ex vivo* significantly. It appears that these mutant RNAs failed to bind some *trans*-acting factors (p37, p41/44 etc) which might be critical for the IRES function. By super-shift assay using anti hnRNPC1/C2 antibody, we have demonstrated that the TSM mutant showed reduced binding to this protein factor. Partial knockdown of hnRNP C1/C2 showed significant decrease in p53 IRES activity and reduced synthesis of ΔN-p53. Also we have showed that introducing compensatory mutations in TSM mutant RNA rescued the secondary structure as well as function of p53 IRES. Further, the role of another silent point mutation in the coding sequence of p53 was investigated. Silent mutation (CCG to CCA) at codon 36 (P36P) showed decreased IRES activity. The mutation also resulted in differential binding of cellular proteins. Taken together, our observations suggest pivotal role of some specific *trans* acting factors in regulating the p53-IRES function, which in turn influences the synthesis of different p53 isoforms.

In the second part of the thesis, p53 IRES RNA interacting proteins were identified using RNA affinity approach. Annexin A2 and PTB associated Splicing Factor (PSF/SFPQ) were identified and their interaction with p53 IRES RNA in vitro and ex vivo was studied. Interestingly, in the presence of Ca^{2+} ions Annexin A2 showed increased binding with p53 IRES. By competition UV crosslinking we have showed Annexin A2 and PSF interact

specifically with p53 IRES. Toe printing assay results showed the putative contact points of Annexin A2 and PSF proteins on p53 IRES RNA. Interestingly, both proteins showed extensive toe-prints in the neighbourhood of the initiator AUG region of p53. Further, competition UV-crosslinking reveals the interplay of these two proteins. Annexin A2 and PSF appear to compete each other for binding with p53 IRES. PSF is known to interact with PTB protein. Since PTB also interacts with p53 IRES and positively regulates the translation, we wanted to study the interplay between PTB and PSF proteins binding with p53 IRES. To address this, we have performed competition UV crosslinking experiment and showed that increasing concentrations of PTB decreases PSF and p53 IRES interaction. However, increasing concentrations of PSF does not decrease or increase in PTB p53 IRES interaction. Results suggest that both Annexin A2 and PSF proteins play important role in regulation of p53 IRES activity.

To address the physiological role of Annexin A2 and PSF proteins on p53 IRES activity, these proteins were partially knocked down in cellulo. This in turn showed decrease in p53 IRES activity in dual luciferase assays as well as in the steady state levels of both the p53 isoforms in transient transfection experiments. Heightened or continued expression of p53 protein is very important under stress where IRES-dependent translation supersedes normal cap-dependent translation. Results showed that expression of Annexin A2 under doxorubicin and thapsigargin induced stress are important for maintenance of both p53 IRES activity and steady state levels of p53 isoforms. Earlier from our laboratory we have showed that the IRES responsible for ΔN-p53 translation is active at G1/S phase while the IRES responsible for full length p53 translation is active at G2/M phase. Subcellular localization of the *trans*-acting factors plays a pivotal role in regulation of IRES activity of cellular mRNA. In this context we wanted to study the nuclear and cytoplasm localization of Annexin A2 under different cell cycle stages. We have seen Annexin A2 protein is dispersed in nucleus

and cytoplasm at G1/S boundary, but post-G2 phase it moved from nucleus to cytoplasm. Further we wanted to investigate the effect of Annexin A2 and PSF on expression of p53 transactivated genes. Partial knock down of Annexin A2 and PSF proteins showed decrease in p21 luciferase activity. By real-time PCR analysis, we have also showed decrease in expression of different p53 targets upon silencing of Annexin A2 protein.

Taken together, our observations suggest pivotal role of *cis* acting and *trans*-acting factors in regulating the p53-IRES function, which in turn influences the synthesis of p53 isoforms.